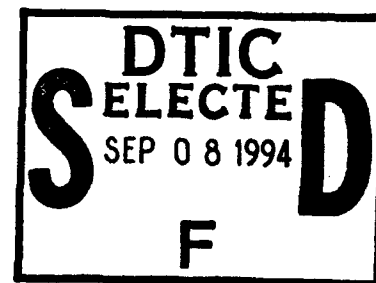# AD-A284 195

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PRINCIPAL INVESTIGATOR: Linda L. Nolan, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts
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Leishmaniasis, a disease caused by protozoan parasites of the Leishmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease are either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis.

The objective of this research was to: 1) Determine the action of the antimalarial 8-aminoquinolines on Leishmania sp., 2) To test the effect of natural products on Leishmania sp. and to 3) Continue the mode of action studies on the nature of the inhibitory action of arachidonic acid on DNA polymerase.

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INTRODUCTION

Leishmania is caused by protozoan parasites of the Order Kinetoplastida: Family - Trypanosomatidae. The disease is estimated to affect 12 million people in Third World countries. Leishmania extracellular forms (promastigotes) are injected into human skin during bites by the sandfly vector. Promastigotes are phagocytized by reticuloendothelial cells, within which the parasites transform into intracellular amastigotes. Human disease results from multiplication of amastigotes within macrophages. Present therapy with pentavalent antimony is potentially toxic, and often ineffective. One rationale for searching for alternative treatment is to identify a unique enzyme system and to target this system for chemotherapeutic exploitation.

Our laboratory is currently studying the mode of action of several antileishmanial agents which demonstrate low toxicity to human cells in order to identify unique target systems.

The specific aims of our current research include:

1) Determining the action of the antimalarial 8-aminoquinolines on *Leishmania sp.*

2) Testing the effect of natural products on *Leishmania sp.*

3) Continuing the mode of action studies on the nature of the inhibitory action of arachidonic acid on DNA polymerase.

THE EFFECTS OF 8-AMINOQUINOLINES ON *Leishmania*

INTRODUCTION

Chloroquine, a 4-aminoquinoline compound, has been the most used antimalarial drug for about 40 years. Today, many malaria strains demonstrate resistance to chloroquine and other antimalarial drugs. In the past 20 years, more and more U. S. civilians were infected by malaria. Recently, to combat the problem of drug-resistance, the 8-aminoquinoline drugs have been developed. These drugs have strong activity against the wild types and some multi-drug-resistant stains. Now three 8-aminoquinoline compounds, WR006026, WR238605 and WR242511, are candidates for antimalarial drugs.

Leishmania species, which cause parasitic protozoal diseases endemic in 80 countries with 350 million people at risk, 12 million infected and an annual incidence of 3-4 million, share some common biochemical characteristics with malarial organisms.

For these reason, our laboratory tested various 8-aminoquinolines for the purpose of assessing their potential as anti-leishmanial agents. Microplate and test tube assays developed in our laboratory (Nolan and Bouchard, 1991) were used to test the inhibitory effect of WR006026, WR238605 and WR242511. The IC_{50} (concentration at 50% inhibition) of these compounds were 50, 4.0 and 2.5 μM respectively. The IC_{50} of these compounds to the human lymphocyte CEM T_4 cells were 60, 5.0 and 5.0 μM respectively. The possible mode of action of these compounds are currently being explored and will be discussed.

Many of the enzymes involved in the metabolism of *Leishmana* have been found to be unique, and for this reason most of the biochemical investigations have been undertaken in the hope of finding compounds which can affect these unique molecules or pathway for chemotherapeutic exploitation.

Leishmania shares many common biochemical characteristics with malarial organisms. Both of them are protozoa, transmitted by insects and infecting human blood cells. Therefore, some of the antimalarial drugs are being screened for their antileishmanial activity.

The antimalarial activity of 8-aminoquinoline compounds was discovered by German scientists during the World War I. 8-aminoquinoline compounds were developed for substitutes for the quinine during the war. Unfortunately, this research became less intense after the war. Chloroquine, a 4-aminoquinoline compound, have been the most used antimalarial drug for about 40 years. Today, many malaria strains demonstrate resistance to chloroquine and other antimalarial drugs. In the past 20 years, more and more U.S. civilians are infected by malaria. Recently, to combat the problem of drug-resistance, the 8-aminoquinoline compounds are being investigated further. These drugs have strong activity against the wild types and some mulit-drug-resistant strains.

The mode of action of these quinoline-containing antimalarial compounds is still unkown. So far, four hypothesis have been reported: 1) DNA Intercalation. The evidence of intercalation is that these compounds can specifically bind to poly (dG-dC) DNA sequnce at low salt concentration. These compounds might be toxic to the parasite by selectively accumulating in specific genes and inhibting their expression (Krogstad and Schlesinger, 1987); 2) Lysosome accumulation. These compounds can diffuse across lysosome membrances. Then they will be protonated and accumulated in lysosome of malaria but not in human blood cells (Ginsburg and Krugliak, 1988); 3) Ferriprotoporphyrin IX (FPIX). Parasites digest human hemoglobin to get nutrition and relearse large amounts of the toxic FPIX. The quinoline-containing compounds can bind with FPIX and causing it to be accumulated in parasite acid food vacules and starving the parasites (Fitch, 1986); 4) Inhibition of heme polymerase. Quinoline-containing compounds inhibit heme polymerase that polymerizes and detoxifies FPIX (Slater and Cerami, 1992; and Chou and Fitch, 1992). The later hypothesis is the most favored.

In this research, three 8-aminoquinoline compounds obtained from Walter Reed Army Institute of Research were studied on their antileishmanial activities. Also the toxicities of these compounds were determined by human CEM T4 *in vitro* assay, developed in our labortory.

MATERIALS AND METHODS

1. 8-AMINOQUINOLINES

Three 8-aminoquinoline compounds (WR006026, and WR238605 and WR242511) obtained from Dr. Joan Jckson, Walter Reed Army Institute of Research, were tested in this experiment.
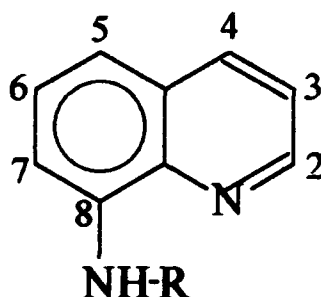
8-Aminoquinolines

2. CELL LINES

Leishmania mexicana 227 was obtained from Dr. Joan Jackson, Walter Reed Army Institute of Research. Cells were maintained in Steiger and Black media (Steiger and Black, 1980). Toxicity towards human cells was assessed by utilizing human lymphocyte CEM T_4 cells, maintained in RPMI 1640 media (GIBCO BRL).

3. INHIBITION TEST (MICROPLATE ASSAY)

Inhibition of the gowth of *Leishmania mexicana* 227 by 8-aminoquinoline compounds was tested on 96-well microplates. Each microwell contained the followinng:

Contents	Blank	Control	Test
Steiger and Black media	300 µl	-	-
L. mexicana 227 cell (10^5 cells/ml)	-	300 µl	300 µl
Solvent of 8-aminoquinoline	-	3.0 µl	-
8-aminoquinoline solutions	-	-	3.0 µl

Experiments were performed in triplicate. The range of concentration of the 8-aminoquinolines tested was 25 nM to 250 µM. The microplates were incubated at 25°C. Cell turbidity (at 660 nm) was monitored on a Microplate Reader (Molecular Devices) at 0, 24 and 48 hours.

4. TOXICITY TEST (TEST TUBE ASSAY)

The toxicity of 8-aminoquinoline compounds on Human lymphocyte CEM T_4 cells was tested using a test tube assay. Each tube contained the following:

Contents	Blank	Control	Test
RPMI 1640 media	5.0 ml	-	-
Human CEM T_4 cells (10^5 cells/ml)	-	5.0 ml	5.0 ml
Solvent of 8-aminoquinoline	-	50 µl	-
8-aminoquinoline solutions	-	-	50 µl

Experiments were performed in duplicate. The range of concentration of the 8-aminoquinolines tested was 25 nM to 250 µM. They were incubated in 5% CO_2 at 37°C. The cell turbidity (at 660 nm) was monitored on the SPECTRONIC 21 (Bausch & Lomb) at 0, 24 and 48 hours.

All tested 8-aminoquinolines inhibited the growth of the *Leishmania mexicana* 227 cells. WR242511 had the strongest inhibition against *Leishmania* while WR006026 had lower inhibition compared with the other compounds.

These 8-aminoquinolines also inhibited the growth of human lymphocyte CEM T_4 cells. WR006026 was less toxic compared with the others.

For all the tested 8-aminoquinolines, IC_{50} to *Leishmania mexicana* 227 were relatively lower than human CEM T_4. This indicates that all these compounds are more toxic to *Leishmania* cells than human cells.

Table. IC_{50} of 8-aminoquinolines to *L. mexicana* and Human CEM T_4

IC_{50}	WR006026	WR238605	WR242511
L. mexicana 227	50 µM	4.0 µM	2.5 µM
Human CEM T_4	60 µM	5.0 µM	5.0 µM

IC50 of 8-Aminoquinolines to L. mexicana 227 and Human T4 Cells
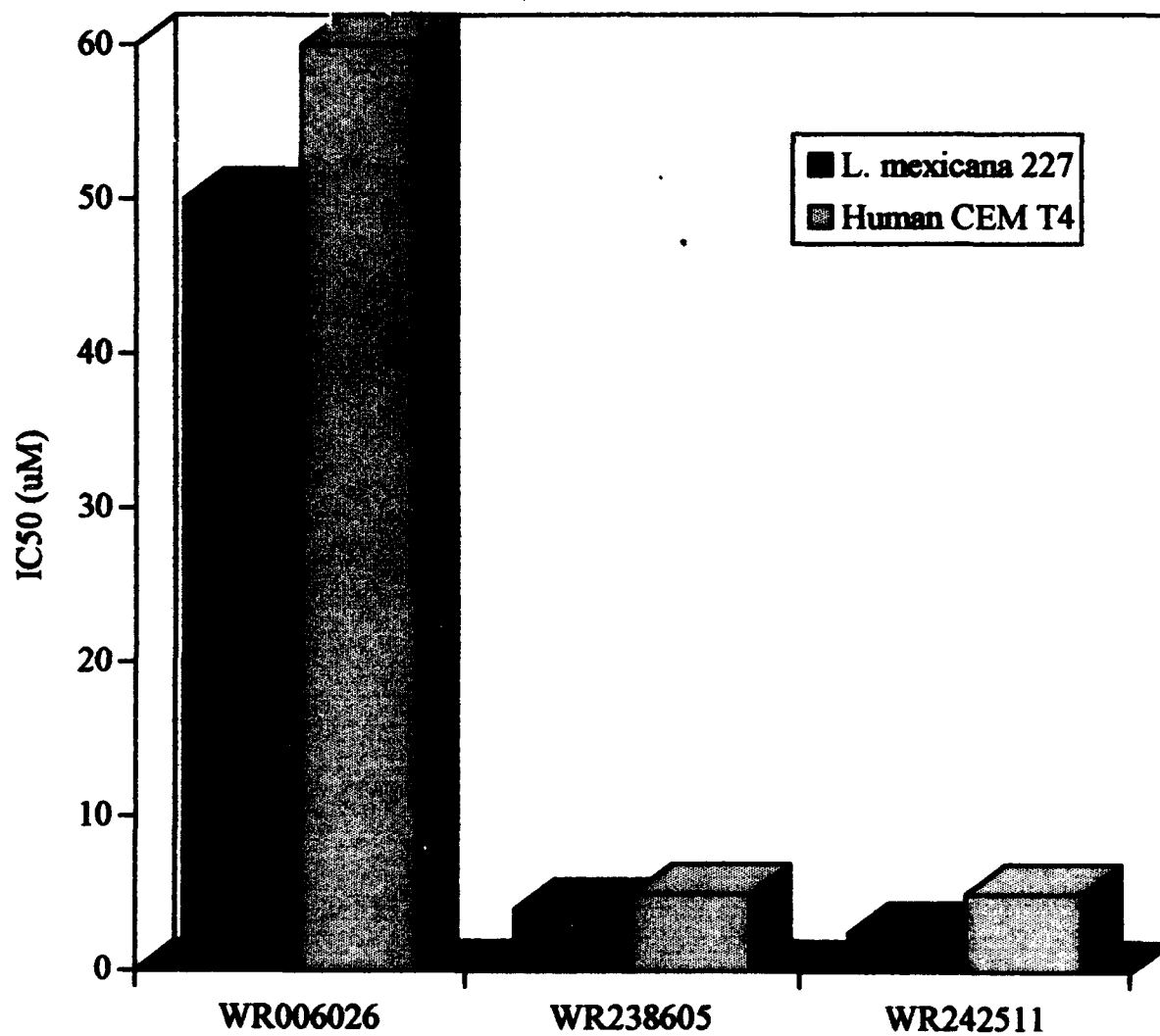
The results of these experiments demonstrates that the 8-aminoquinoline compounds strongly inhibit the growth of *Leishmania* cells at very low concentrations. At higher concentrations, they are toxic to human cells.

Unlike malaria cells, the main food source of *Leishmania* cells is not the human hemoglobin. Therefore, the heme polymerase could not be the molecular target of 8-aminoquinolines in *Leishmania* cells. 8-aminoquinolines in *Leishmania* might have the same mode of action as in malaria, such as intercalate with *Leishmania* DNA and accumulation in lysosome, or it might attack other molecules. The mode of action of these 8-aminoquinoline compounds in *Leishmania* cells is currently being ivestigated in this laboratory.

New modified 8-aminoquinoline will be synthesized and tested, which may increase the inhibition to *Leishmania* and decrease the toxicity to human. 8-aminoquinolines have potential to be developed for the chemothyrapy of leishmaniases and become new antileishmania drugs.

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Effect of *Allium sp.* and Other Natural Herb Extracts on the Growth of *Leishmania sp.* and Mammalian Cell Lines.

Various medicinal, insecticidal, antibacterial, antiprotozoal, and antifungal properties have been ascribed to natural herbs. Leishmanial protozoal parasites and a mammalian cell line, whose sensitivity to natural herbs was undetermined, were tested for their susceptibility to aqueous and ethanol plant extracts including; nutmeg *(Myristicaceae sp.)*, ginger *(Zingiber officinale)*, goldenseal root *(Hydrastis canadensis)*, garlic *(Allium sativum)*, elephant garlic *(Allium scorodoprasum)*, onion *(Allium cepa)*, and licorice *(Glycyrrhiza glabra)*. Cells of *Leishmania mexicana 227* and *Leishmania chagasi 13* were cultured in nutrient media MM2. Growth was monitored turbidimetrically at 590 nm in micro well plates with the help of a Molecular Devices THERMOmax microplate reader. Measurements were recorded after a 72 hour incubation period. HeLa cells were cultured in RPMI-1640 media supplemented with 5% fetal bovine serum. The percent inhibition of the *in vitro* growth of mammalian cells indicated the toxicity of the herb toward humans. Results demonstrated that *L. chagasi 13* was more sensitive to both the elephant garlic *(Allium scorodoprasum)* and the household garlic *(Allium sativum)* than the *L. mexicana 227*. The extract from raw onion *(Allium cepa)* did not inhibit cell growth for any of the cell lines. Extracts of licorice *(Glycyrrhiza glabra)* inhibited the growth of the leishmanial parasites, but were not toxic to HeLa cells. All of the extracts showed varying inhibitory activities. Results confirmed the previously known antiprotozoal activity of garlic *(A. sativum, A. scorodoprasum)* and demonstrated the inhibitory properties of the other herbs.

Our laboratory is investigating potential antileishmanial agents. The three forms of leishmaniasis, cutaneous, mucocutaneous and visceral, affect over 12 million people worldwide. The disease is especially endemic to the regions of Southern Asia, Northern Africa, Central and South America (1). The present chemotherapeutic agents used in the treatment of leishmaniasis are extremely toxic. The exorbitant cost and lack of availability of these compounds to the endemic regions make them ineffective and inefficient (2).

Our ancestors have been benefitting from the properties of natural products since early times. The Indigenous people of the Americas used goldenseal to battle infections long before their encounter with Columbus (3). Licorice has been used as a remedy against bronchitis and certain cancers (4). Priests, during the Middle Ages, wore garlic necklaces to protect themselves from the Bubonic Plague (5). The vast documentation of the value of these herbal medicines throughout history, sparked our current investigations for testing their potential for antileishmanial activity.

The purpose of this study was to evaluate the effects of seven known antimicrobial herbs on the *in vitro* growth of the protozoal parasites, *Leishmania sp.* and mammalian HeLa cells. Cell growth measurements were recorded by optical analysis at 590 nm for *Leishmania sp.* and at 490 nm for HeLa cells. Recorded growth measurements were after a seventy-two hour incubation period. The antimicrobial herbs were tested against *L. mexicana 227*, *L. chagasi 13*, and HeLa cells. The plant extracts included; *Allium cepa* (onion), *Allium scorodoprasum* (elephant garlic), *Allium sativum* (garlic), *Zingiber officinale* (ginger), *Myristicaceae sp.* (nutmeg), *Hydrastis canadensis* (goldenseal root) and *Glycyrrhiza glabra* (licorice).

Materials & Methods

Cultures of Parasitic Protozoa: Promastigotes of *Leishmania mexicana* Walter Reed strain 227 and *Leishmania chagasi* Walter Reed strain 13 were maintained in Corning culture flasks. The cells were obtained from Dr. Joan Jackson (Walter Reed Army Institute of Research). The protozoan were cultured in defined medium MM2. The protozoan parasites were grown at 25°C and subcultured twice weekly in an O_2 incubator.

Cultures of Human HeLa Cells: Human cervical cancer cells obtained from the Department of Veterinary and Animal Science at the University of Massachusetts-Amherst. The cells were cultured in Corning tissue flasks with RPMI 1640 medium. Cultures were incubated in a 5% CO_2 chamber at 37°C and subcultured twice weekly.

Preparation of Natural Product Extracts: The dried herbs were put into either Super-Q deionized distilled water or 95% ethanol suspension. The fresh *Allium sp.* did not require hydration. Both the dried and fresh herbs were blended with a Waring blender at the maximum speed. The herbal suspension was then spun down in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Dupont Instruments, Newtown, CT) at 20,000 RCF. The supernatant was separated from the pellet and sterilized through a Corning 0.45 um sterile filter apparatus (Fischer Scientific, Pittsburgh PA)

All extracts were quantitated utilizing a Bio-Rad Protein Determination Assay after the completion of the sterilization process. Various concentrations of the extracts were tested in the assay system to determine the protein concentration that causes a 25 and 50 percent inhibition of in *vitro* cell growth (IC25 and IC50).

Protein Assay Procedure: Protein concentrations were determined using the dye-binding method (Bio-Rad Laboratory, Hercules, CA). The method was modified and performed in a Falcon 3071 Microtest III tissue plates (Fischer Scientific, Pittsburgh, PA). 100 ul of Bio Rad dye was added to 100 ul of diluted extract. The plate was read in a Microsoft THERMOmax microplate reader. The turbidity was measured at 590 nm.

Assay Inoculum: *Leishmania sp.* and HeLa cells were diluted with fresh medium twenty-four hours prior to testing to ensure a log-phase culture. The inoculum was standardized at the zero hour of the assay with a Spectronic 21 spectrophotometer (Bausch & Lomb, Rochester NY) in order to eliminate variations caused by different concentrations of cells growing at varying rates. This procedure standardized the inhibition assay.

Microplate Assay Procedure: Assays were performed in Falcon 3071 Microtest III tissue culture plates. These plates are 96 well, flat bottom, tissue culture, gamma irradiated, polystyrene plates with low evaporation lids. The wells were monitored turbidimetrically over a period of 72 hours with a Microsoft THERMOmax microplate reader.

Measurement of Toxicity: Toxicity to cells was measured by the protein concentration of an agent that causes 25 and 50 percent inhibition in the in *vitro* cell growth relative to the untreated control cells. Percent inhibition was calculated according to the following formula:

$$\left[\ 1-\ \underline{\text{cell growth in test wells}}\ \right]\ X\ 100$$
$$\text{cell growth in control wells}\quad .$$

The percent inhibitions were graphed versus the respective protein concentrations. The IC25 and IC50 were calculated from the graphs. Concentrations of herbs at the IC25 and IC50 were retested to ensure accuracy. Comparison of the inhibitory concentrations between the mammalian and leishmanial cell lines indicated the potential of the herb as an anti-leishmanial agent.

All data were entered on an Apple Macintosh IIsi using a Microsoft Excel spreadsheet program.

RESULTS

The IC25 and IC50 of the natural herbal extracts towards the individual cell lines are shown in Table 1-3.

Garlic extracts of *Allium sativum* (Fig.1) and *Allium scorodoprasum* (Fig.2) exhibited the most inhibitory activity against *Leishmania chagasi 13*. The IC50s were 76 ug protein/ml and 32 ug protein/ml respectively.

The *allium sp.* extracts demonstrated similar inhibition towards *Leishmania mexicana 227*. The IC50 for the *A. sativum* (Fig 3) extract was 105 ug protein/ml and the *A. scorodoprasum* (Fig.4) extract exhibited an IC50 of 100 ug protein/ml.

The *A. sativum* (Fig.5) inhibitory activity was similar for HeLa cells (IC50: 76 ug protein/ml), but the *A. scorodoprasum* (Fig.6) was less inhibitory to the HeLa cells (no IC25 recorded).

Only three herbal extracts demonstrated inhibitory properties to *L. mexicana 227*. They were *Glycyrrhiza glabra* (IC25: 6 ug protein/ml)(Fig.7), *Zingiber officinale* (IC25: 14 ug protein/ml), and ethanol extracts of *Myristacea sp.* (IC25: 31 ug/ml).

More herbal extracts exhibited inhibition towards *L. chagasi 13*, than *L. mexicana 227*, including both aqueous *G. glabra* (IC50: 85 ug protein/ml)(Fig.8) and ethanol *G. glabra.* (IC50: 240 ug protein/ml). An IC25 was obtained with ethanol extracts of *Hydrastis canadensis* (IC25: 314 ug protein/ml), aqueous extracts of *Z. officinale* (IC25: 80 ug protein/ml), ethanol extracts of *Z. officinale* (IC25: 42 ug protein/ml), and aqueous extracts of *Myristacea sp.* (IC25: 4.25 ug protein/ml)

The HeLa cells were less sensitive to the herbs tested. Ethanol extracts of *G. glabra* demonstrated an IC25 of 179 ug/ml. Also, aqueous extracts of *H. canadensis* (IC25: 13 ug protein/ml) and of *Myristacea sp.* (IC25: 2.7 ug protein/ml) demonstrated inhibitory activity. Aqueous and ethanol extracts of *Z. officinale*, aqueous *G. glabra*, ethanol extracts of *H. canadensis*, ethanol extracts of *Myristacea sp.* and *A. cepa* demonstrated no IC25s.

Many extracts demonstrated weak or no inhibitory activity (Table 4). IC25s towards *L. chagasi 13* were not demonstrated for; aqueous extracts of *G. glabra*, aqueous *A. cepa*, ethanol extracts of *Myristacea sp.*, and aqueous *H. canadensis*. Also, IC25s could not be demonstrated towards *L. mexicana 277* for; extracts of *A. cepa*, ethanol extracts of *G. glabra*, ethanol and aqueous extracts of *H. canadensis*, and ethanol extracts of *Z. officinale*.

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Table 1						
Summary of Inhibitory Activity of Natural Herb Extracts						
On the Growth of		*Leishmania chagasi*				
Natural Product	**Solvent**		**Protein concentration**	**Percent Inhibition**	**IC25**	**IC50**
			Range (ug/ml)	Range	(ug/ml)	(ug/ml)
Glycyrrhiza glabra	AQ	1	15-150	7.33-53.21	22	85
(Licorice)	ETOH	2	0.26-268	16.85-53.93	15*	240
Hydrastis canadensis	AQ		5-15.0	0.0-5.51	none	none
(Goldenseal Root)	ETOH		0.3-10.0	20.22-33.06	314	none
Zingiber officinale	AQ		15-200	29.35-39.44	80	none
(Ginger)	ETOH		0.2-233	15.93-40.44	42	none
Myristaces sp.	AQ		1-4.0	9.17-23.85	4.25*	none
(Nutmeg)	ETOH		4.0-408.5	12.36-16.85	none	none
Allium sativum	AQ		12-1200	7.21-90.77	35	76
(Garlic)						
Allium scorodoprasum	AQ		0.3-34	3.07-52.0	12	32
(Elephant Garlic)						
Allium cepa	AQ		2.17-217	4.61-10.72	none	none
(Onion)						
* Estimated						
1 AQ= Aqueous extraction						
2 ETOH= Ethanol Extraction						

Table 2					
Summary of Inhibitory Activity of Natural Herb Extracts					
		On the Growth of .	*Leishmania mexicana*		
Natural Product	Solvent	Protein concentration	Percent Inhibition	IC25	IC50
		Range (ug/ml)	Range	(ug/ml)	(ug/ml)
Glycyrrhiza glabra	AQ	15-150	26.37-41.75	6*	none
(Licorice)	ETOH	0.26-268	No Inhibition	none	none
Hydrastis canadensis	AQ	5-15.0	0.0-13.19	none	none
(Goldenseal Root)	ETOH	0.3-30.4	No Inhibition	none	none
Zingiber officinale	AQ	15-200	27.47-38.46	14*	none
(Ginger)	ETOH	0.2-233	No Inhibition	none	none
Myristacea sp.	AQ	1-4.0	18.68-20.88	none	none
(Nutmeg)	ETOH	4.0-40.85	1.51-34.85	31	none
Allium sativum	AQ	12-1200	0.0-93.0	64	105
(Garlic)					
Allium scorodoprasum	AQ	3-121	0.0-64.0	47	100
(Elephant Garlic)					
Allium cepa	AQ	2.2-222	No Inhibition	none	none
(Onion)					
* Estimated					

Table 3					
Summary of Inhibitory Activity of Natural Herb Extracts					
	On the Growth of Mammalian HeLa Cells				
Natural Product	**Solvent**	**Protein concentration**	**Percent Inhibition**	**IC25**	**IC50**
		Range (ug/ml)	**Range**	**(ug/ml)**	**(ug/ml)**
Glycyrrhiza glabra	AQ	15-150	No Inhibiton	none	none
(Licorice)	ETOH	2.6-268	10-32.25	179	none
Hydrastis canadensis	AQ	5-15.0	0.0-30.12	13	none
(Goldenseal Root)	ETOH	0.3-30	10.0-19.01	none	none
Zingiber officinale	AQ	15-200	2.4-15.01	none	none
(Ginger)	ETOH	0.23-23	No Inhibition	none	none
Myristacea sp.	AQ	1-4.0	0.0-34	2.7	none
(Nutmeg)	ETOH	4.0-408.5	No Inhibition	none	none
Allium sativum	AQ	12-1200	9.23-90.77	34	76
(Garlic)					
Allium scorodoprasum	AQ	0.3-330	0.0-16.92	none	none
(Elephant Garlic)					
Allium cepa	AQ	0.2-22	0.0-10.72	none	none
(Onion)					

Table 4		•	
Herbs Demonstrating Weak Inhibition			
vs HeLa Cells		**Protein Concentration**	**Percent Inhibition**
Glycyrrhiza glabra	AQ (Licorice)	15-150 ug/ml	0.0%-7.9%
Zingiber officinale	AQ (Ginger)	15-200 ug/ml	0.0%-6.65%
Myristiceae sp.	ETOH (Nutmeg)	4-40 ug/ml	0.0%-5.0%
vs *Leishmania mexicana*			
Allium cepa	AQ (Onion)	2.2-223 ug/ml	No Inhibition
Glycyrrhiza glabra	ETOH (Licorice)	15-150 ug/ml	No Inhibition
Hydrastis canadensis	ETOH (Goldenseal Root)	0.3-30.4 ug/ml	No Inhibition
Zingiber officinale	ETOH (Ginger)	15-200 ug/ml	No Inhibition
vs *Leishmania chagasi*			
Hydrastis canadensis	AQ (Goldenseal Root)	5-15 ug/ml	0.0%-5.51%

Figure 1

Inhibitory Activity of Aqueous Allium sativum (Garlic)   on the in vitro Growth of L. chagasi
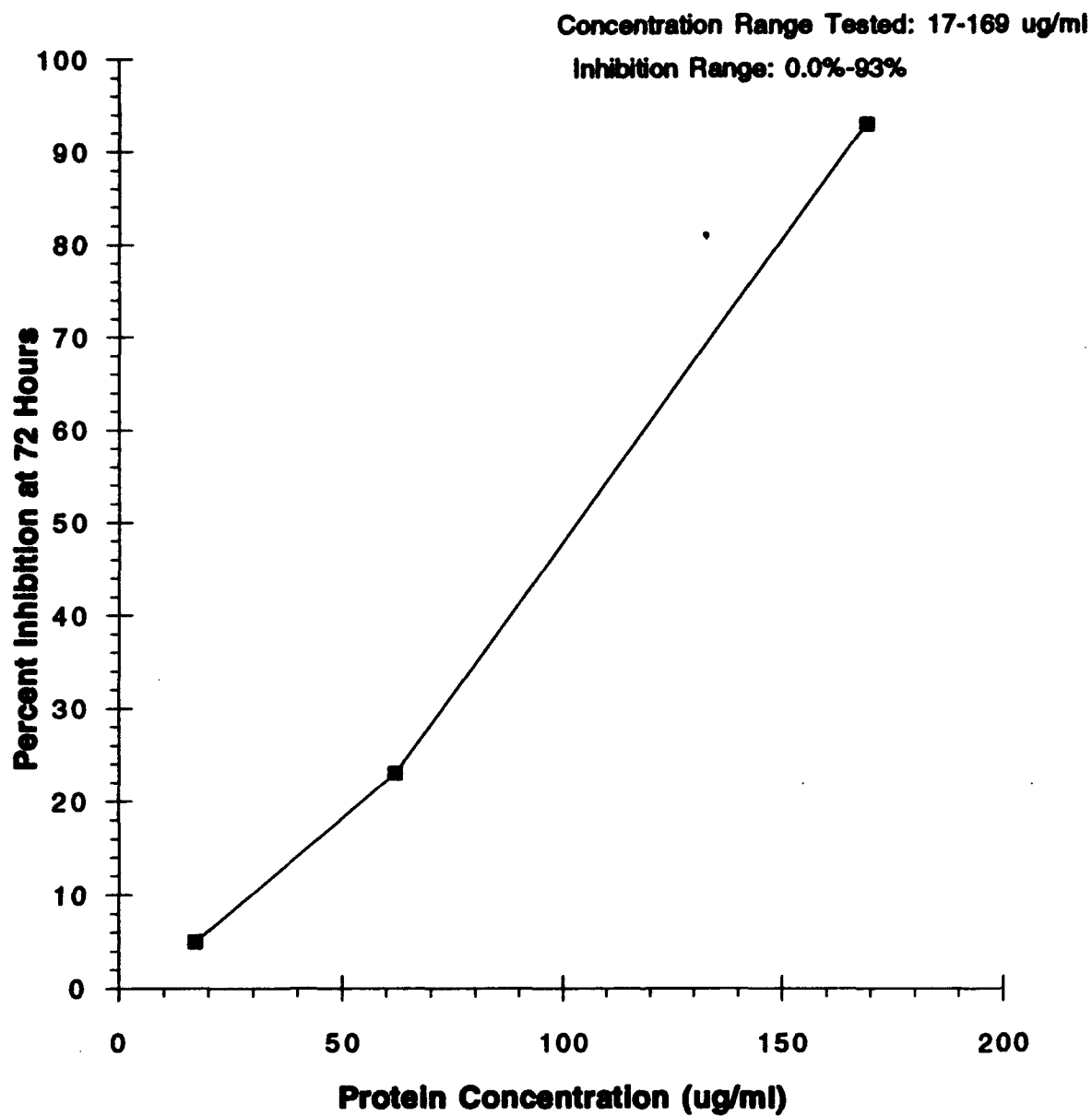
Concentration Range Tested: 17-169 ug/ml
Inhibition Range: 7.21%-90.77%
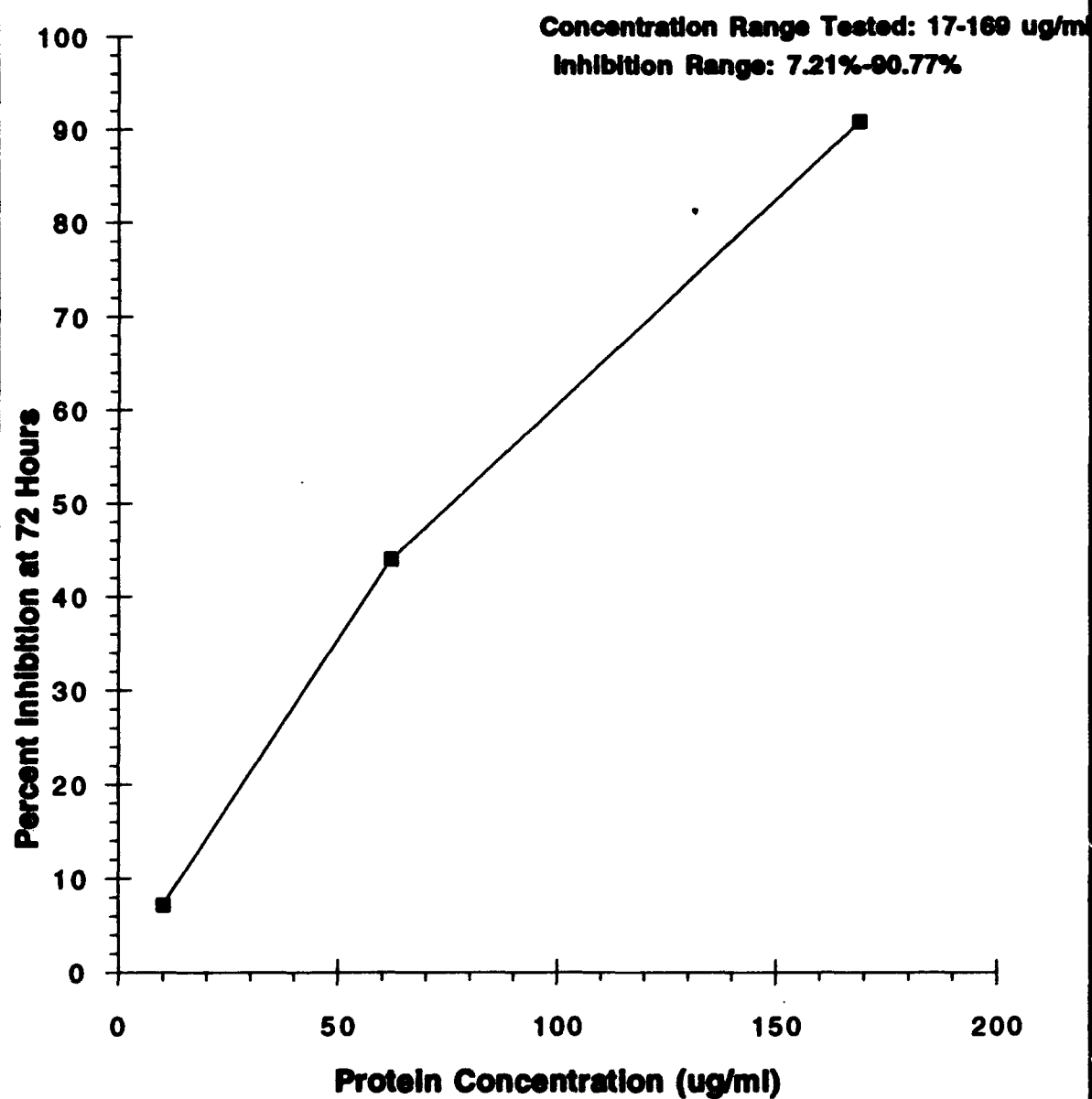
Figure 2

Inhibitory Activity of Aqueous Allium
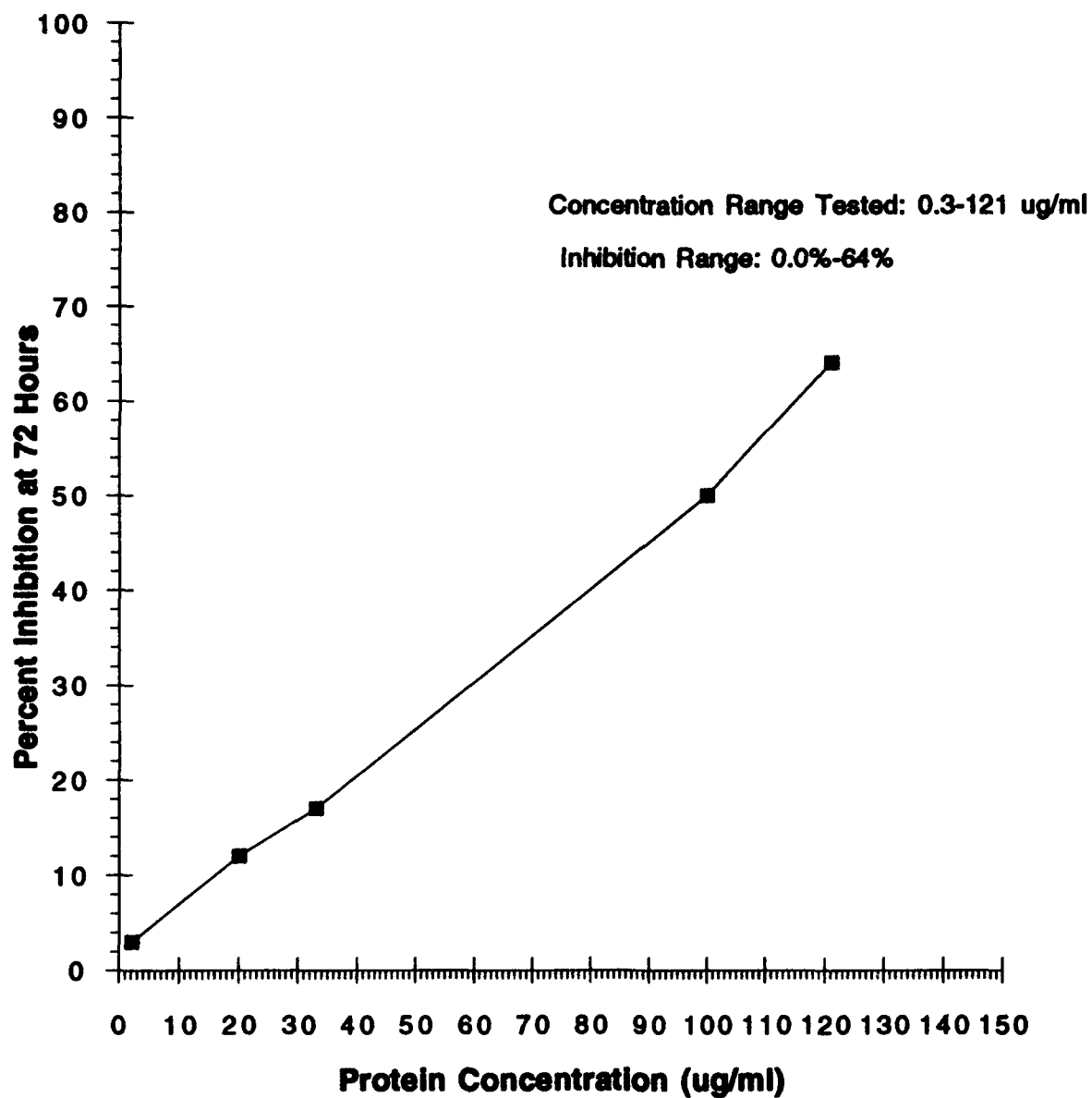scorodoprasum (Elephant garlic) on the in vitro
Growth of L. chagasi 13

Concentration Range Tested: 0.3-34 ug/ml

Inhibition Range: 3.07%-52%
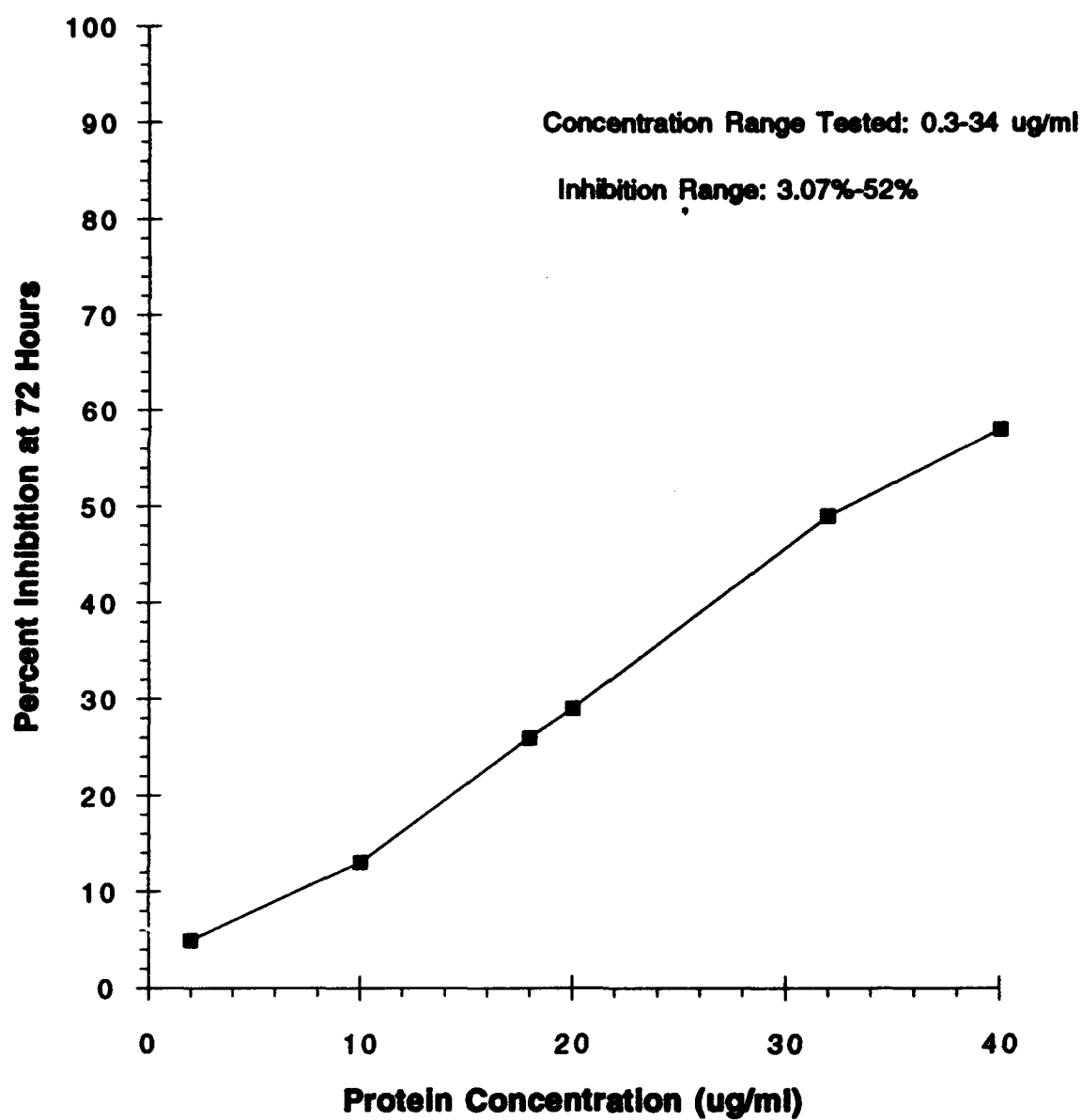
Figure 3

Inhibitory Activity of Aqueous Allium sativum (Garlic)   on the in vitro Growth of L. mexicana

Concentration Range Tested: 17-169 ug/ml
Inhibition Range: 0.0%-93%

20

Figure 4

Inhibitory Activity of Aqueous Allium scorodoprasum (Elephant Garlic) on the in vitro Growth of L. mexicana 227.

Concentration Range Tested: 0.3-121 ug/ml

Inhibition Range: 0.0%-64%

Y-axis: Percent Inhibition at 72 Hours

X-axis: Protein Concentration (ug/ml)

Figure 5

Inhibitory Activity of Aqueous Allium sativum (Garlic) on the in vitro Growth of HeLa Cells

Concentration Range Tested: 12-1200 ug/ml

Inhibition Range: 7.23%-90.77%
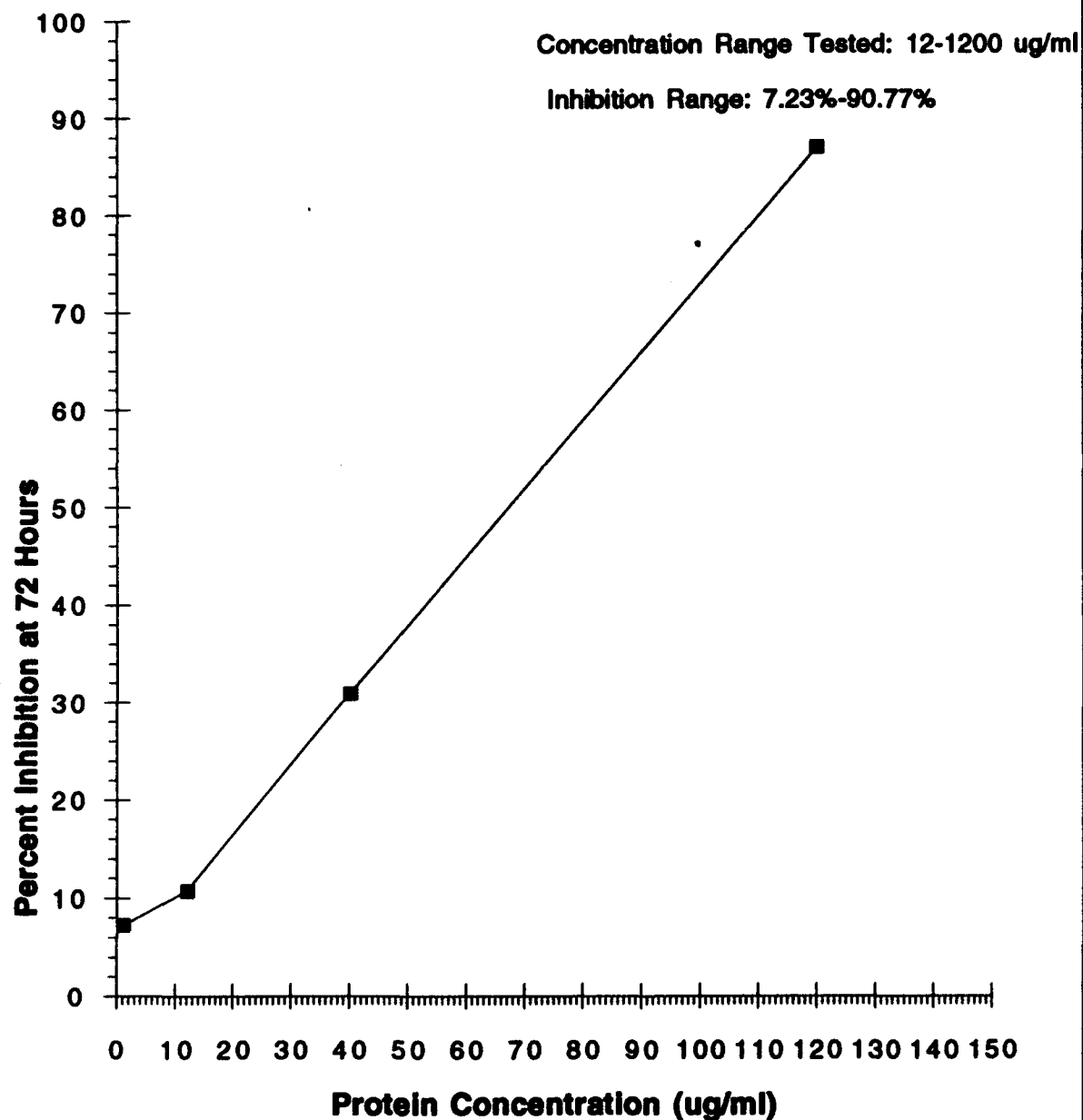
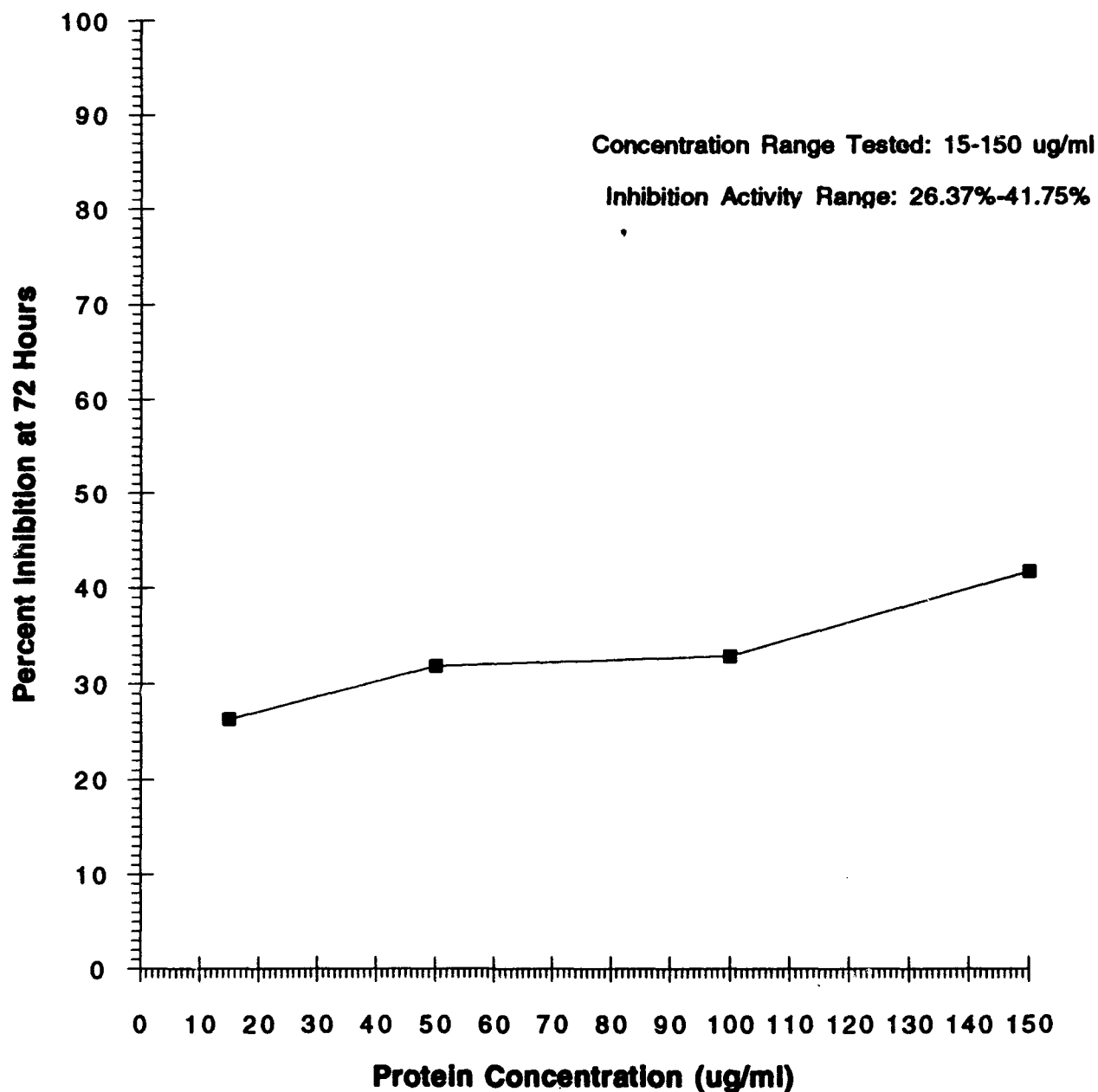**Figure 6**

**Inhibitory Activity of Aqueous Allium scorodoprasum (Elephant Garlic) on the in vitro Growth of HeLa Cells**

Concentration Range Tested: 0.3-330 ug/ml

Inhibition Range: 0.0%-16.92%

23
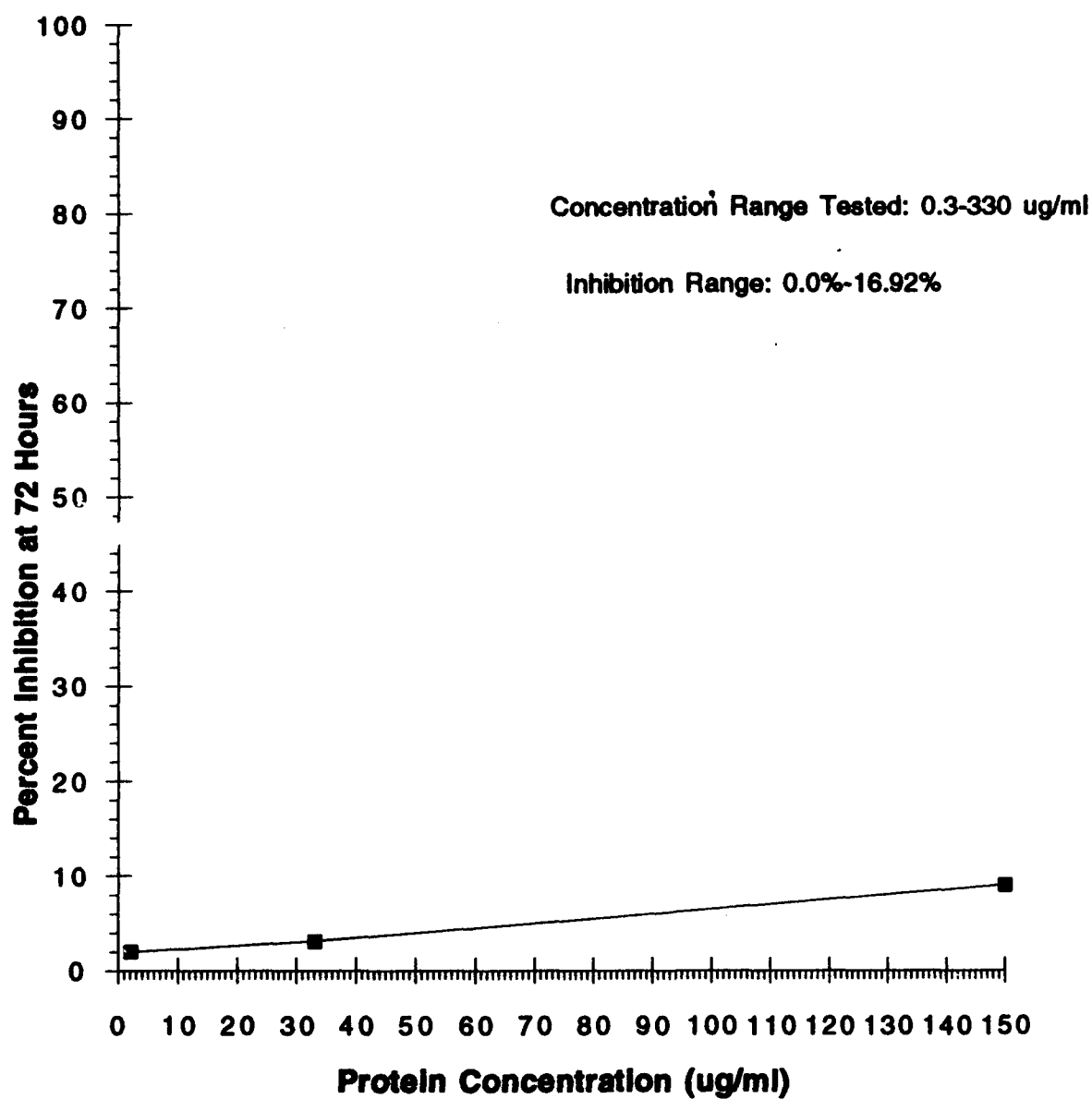
Figure 7 **Inhibitory Activity of Aqueous G. glabra (Licorice) on the in vitro growth of L. mexicana 227**

Concentration Range Tested: 15-150 ug/ml

Inhibition Activity Range: 26.37%-41.75%

X-axis: Protein Concentration (ug/ml)

Y-axis: Percent Inhibition at 72 Hours

Figure 8   **Inhibitory Activity of Aqueous Glycyrrhiza glabra (Licorice) Extract on the in vitro Growth of L. chagasi 13**

Concentration Range Tested: 15-150 ug/ml

Inhibition Activity Range: 7.33%-53.21%
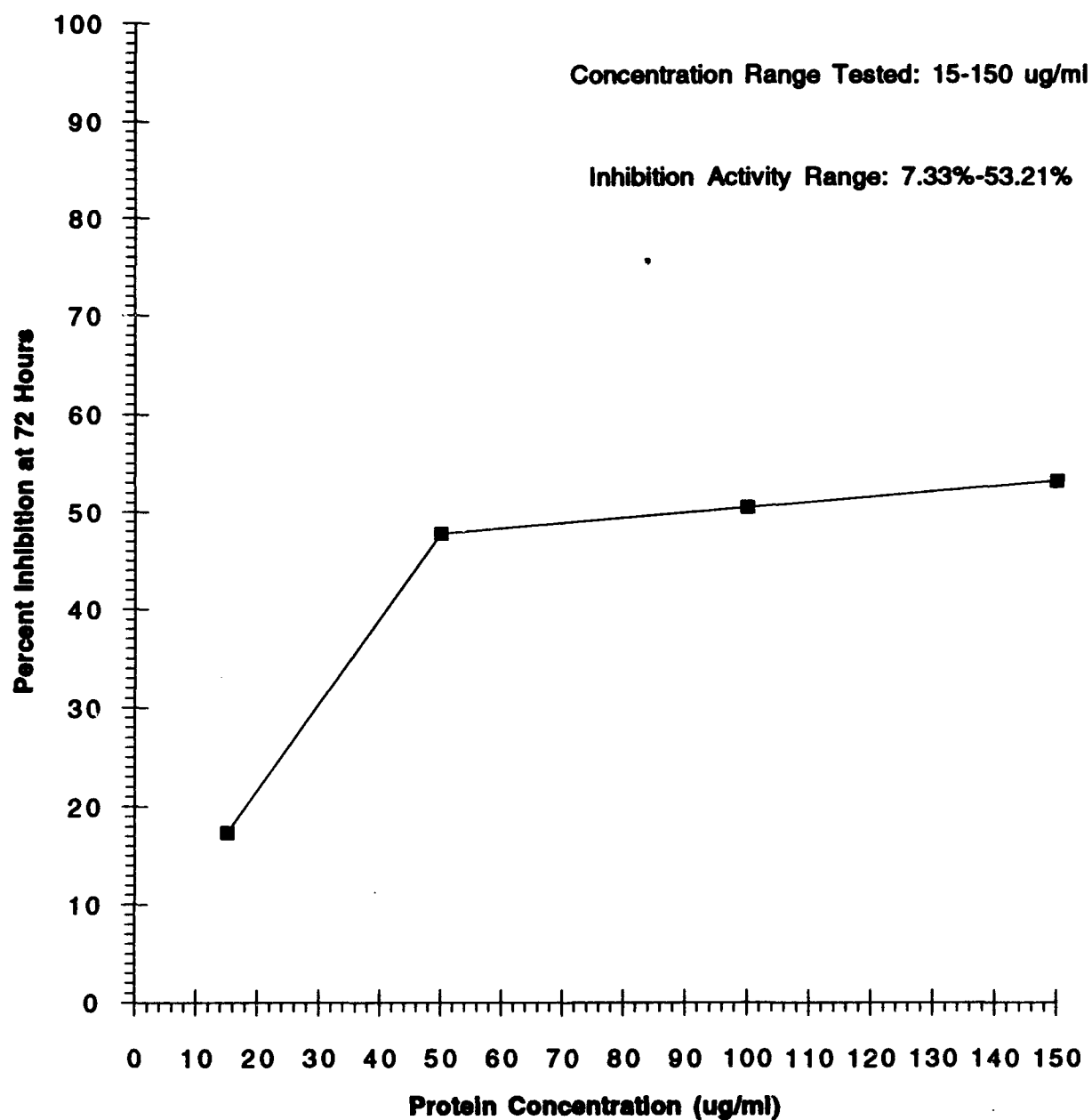
Discussion

The value of any chemotherapeutic agent lies in its selective toxicity for the host. By comparing the inhibitory activities of the natural herbal extracts and the different cell lines, assessment of anti-leishmanial potential of the herbs is possible.

Our preliminary results indicate that of all the herbs tested, only *Glycyrrhiza glabra* (licorice) and *Allium scorodoprasum* (Elephant garlic) exhibited potential as antileishmanial agents.

Aqueous *A. scorodoprasum* extracts exhibited and IC50 of 100 ug/ml against the *Leishmania mexicana 227* and an IC50 of 32 ug/ml against the *L. chagasi13*. At the highest concentration tested, little inhibition was observed with the HeLa cells.

Incorporation of garlic in herbal medicines has been documented since the dawn of civilization. *A. scorodoprasum* is a close relative to the better known *A. sativum*. The bulb of *A. scorodoprasum* has similar morphology but, is six times larger on average than *A. sativum* (6). It also has less of an odor and taste than *A. sativum* due to a lower sulfur compound concentration (7). It has been reported that *A. scorodoprasum* acts as an anti-tumor agent as well as an anti-cancer agent (8). *A. scorodoprasum* has also been reported to be effective against typhoid, amoeboid dysentery and parasitic worms (9). The allium has been classified as an antibacterial, fungicide and a weak antiviral. *A. scorodoprasum* is highly nutritious (10). *A. scorodoprasum* use as a combative for malnutrition, is due to a high concentration of vitamins and essential amino acids (11).

The active ingredient of the *A. scorodoprasum* is thought to be allicin. Allicin is not found in intact garlic cells, but is formed by the enzymatic action of alliin alkyl-sulfenate-lyase (E.C.4.4.1.4) on the non-protein amino acid, S-allylcysteine S-oxide (alliin). The enzyme reaction generates many other secondary products as waste. These include pyruvate, ammonia and allylsulfenic acid (12). Other groups are investigating ajoene, diallyl sulfide, allyl disulfide and diallyl trisulfide as potential active ingredients (13).

The *G. glabra* extract also possesses significant anti-leishmanial qualities. Aqueous licorice extracts exhibited IC25 of 6 ug/ml against the *L. mexicana227* and an IC25 of 22 ug/ml and IC50 of 85 ug/ml against *L. chagasi13*. The aqueous extracts demonstrated no significant inhibition against the mammalian HeLa cells. The ethanol extracts demonstrate IC50s at 240 ug/ml against only *L. chagasi13*. These striking differences in IC50 between the cell lines and the parasite indicate their potential as an anti-leishmanial agents.

G. glabra is a known antibacterial and weak antiviral agent. The medicinally active compound of the root combats bronchitis, gastric ulcers and Addison's disease (14). The active ingredient in the compound is a saponin-like glycoside. The terpene, glycyrrhentic acid, is a hemolytically active compound, and resembles a steroid. Glycyrrhentic acid is used in the manufacturing of carbenoxolone (15). The compound stimulates the adrenal cortex hormone aldosterone. It also has known estrogenic effects (16).

Fractionation of extracts of *G. glabra* and *A. scorodoprasum* are scheduled for testing of further antileishmanial activity. The active components will be identified and tested. Mode of action studies of the active ingredients will be performed.

Since many of the natural products tested have never been examined as anti-leishmanial agents, the results of these experiments are the first to be reported. Further studies will be performed to elucidate the inhibitory components responsible for the anti-leishmanial activity of *A. scorodoprasum* and *G. glabra.*

Inhibition of DNA Polymerase Activity By Arachidonic Acid in Vitro

ABSTRACT

Arachidonic acid has been found to inhibit the activity of DNA polymerases in vitro at concentrations below the critical micelle concentration. The inhibition was found to be specific for DNA polymerase activity, and other enzymes such as alkaline phosphates, trypsin, and DNAse I were not inhibited by the fatty acid. Data suggests a physical association between the enzyme and arachidonic acid. The inhibition was found to be reversible by dialysis of the arachidonic acid from solution. This inhibition could represent novel mechanisms by which the cell regulates the synthesis of DNA in response to mitogenic stimuli, injury, disease, or toxic insult.

Analysis of arachidonic acid in *Leishmania mexicana* promastigates revealed the absence of synthesis of this normal mammalian fatty acid in the parasite.

Since this intracellular parasite elicits the rise of arachidonic acid levels in macrophages during the invasion, this phenomenon could arise from the host cell's response to terminate the infection.

INTRODUCTION

Deoxyribonucleic acid (DNA) replication in mammalian cells involves the activity of at least five distinct DNA polymerases (DNA nucleotidyl transferases, EC 2.7.7.7). These DNA polymerases have been designated in order of discovery as DNA polymerases α, β, γ, δ, and ϵ. All of the DNA polymerases have the same basic catalytic characteristics, such as utilization of deoxyribonucleoside triphosphates for the sequential incorporation of deoxyribonucleoside diphosphates into DNA, with cleavage of a phosphodiester bond and release of pyrophosphate. Synthesis of DNA is template-directed and in addition, requires a metal ion activator and a short sequence of DNA or RNA complementary to the template to act as a primer for synthesis. DNA synthesis is catalyzed only in the 5' $\Rightarrow$ 3' direction of the newly synthesized strand (Fry and Loeb, 1986).

The role of arachidonic acid (AA) in the cell is usually considered to be as a precursor to potent biological molecules such as leukotrienes and prostaglandins. Very little attention has been directed to its potential direct involvement in cell metabolism. Extensive research during the last three decades has shown that AA is a versatile molecule that affects a multitude of cellular processes, either directly or through its metabolites. The bulk of AA in mammalian cells is found in cell membranes, where it is esterified in the fatty acyl chains of glycerophospholipids. AA is released from the membranes by the actions of phospholipases and lysophospholipases, where it may be reacylated into complex lipids by the action of acyl transferases or it may be metabolized into potent biological products by two enzymatic pathways. The cyclooxygenase pathway results in the generation of prostaglandins, prostacyclins, thromboxanes, and malondialdehyde from AA. The lypoxygenase pathway produces leukotrienes and other eicosanoids such as 5-hydoxyeicosatetraenoic acid (5-HETE) and 5, 12-dihydroxyeicosatetraenoic acid (5,12-DHETE) from AA.

The level of free AA in the cytoplasm and plasma is disproportionately low when compared to its esterified levels. This level represents a balance between hydrolysis of AA from membrane lipids, its esterification back into complex membrane lipids, and its metabolism by enzymes (Irvine, 1982). This balance can be altered by various physiological effectors in some tissues. For example, Morita and Murota (1980) observed that ATP levels could markedly influence the fate of AA in liver homogenates, and suggested that cellular ATP levels may influence the AA release/reacylation balance.

A variety of mechanical, chemical, or hormonal mitogenic stimuli lead to a cascade of reactions that involve activation of membrane-associated phospholipases that release arachidonic acid and other lipids from the membranes. The release of AA from the membranes leads, in turn, to a series of biochemical events that alter the metabolism of the cell and allows, among others, repair processes to occur (Needleman et al., 1986).

An example where lipids mediate metabolic processes has been described by Sylvia and coworkers (Sylvia and coworkers (Sylvia et al., 1986, 1988). These investigators proposed a possible mechanism where DNA replication prior to cell division is affected by phospholipids. A low specific activity DNA polymerase a was activated in vitro by a phosphorylated phosphatidylinositol, supporting their proposal (Sylvia et al., 1988). These authors suggested that a phosphorylation cascade may be involved in the modulation of DNA polymerase a activity prior to the initiation of mitosis. Subsequent work showed that the phosphatidylinositol is apparently hydrolyzed in the presence of a low activity form of DNA polymerase a, resulting in the release of diacylglycerol and the retention of inositol -1, 4-bisphosphate by the enzyme complex (Sylvia et al., 1988).

DNA synthesis studies in which inhibitors of lipoxygenase and cyclooxygenase (two enzymes involved in AA metabolism) were used, showed a dose-response inhibition of [^{3}H] Thymidine incorporation in mouse epidermal cells (Fischer, 1985). In addition, topical application of AA resulted in a dramatic inhibition of tumor production in SENCAR mice at doses over 100 µg (Fischer, 1985).

The available data suggests a possible involvement of fatty acids, specifically of archidonic acid, in the controlling cell metabolism. Our work supports the involvement of arachidonic acid in affecting DNA replication in vitro.

MATERIALS & METHODS

Leishmania mexicana DNA polymerase A (pol A) was isolated as described (Nolan et al., 1992). DNA polymerase α from chinese hamster ovary (CHO pol α) was a generous gift from Drs. Naseema N. Khan and Neal Brown, Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA. HeLa cell DNA polymerase α, β, and γ were a generous gift from Dr. L.M.S. Chang, Uniformed Services University of the Health Sciences, Bethesda, MD. Yeast DNA polymerases α, δ, and ε were a generous gift from Dr. A. Sugino, National Institute of Environmental Health Sciences, Research Triangle Park, NC. A mouse recombinant DNA polymerase β was a generous gift of Dr. S.H. Wilson, U. of Texas at

Galveston. DNA polymerase I from E. coli (pol I), and Micrococcus luteus DNA polymerase (M. luteus pol) were from Pharmacia LKB. Additional M. luteus pol, trypsin, trypsin inhibitor, alkaline phosphatase, and the alkaline phosphatase kit were obtained from the Sigma Chemical . Company

All DNA polymerases were assayed at 35° C for 30 minutes in a final volume of 32 μl in a reaction mixture containing 100 μM each of dATP, dCTP, and dGTP; 50 μg/ml of heat-denatured BSA; 100 μM dithiothreitol, 150 μg/ml activated DNA from calf thymus, and 40 nm [methyl-^{3}H] dTTP. CHO pol α was assayed in 130 mM KPO$_4$ (pH 7.4) and 8mM MgCl$_2$. Pol A was assayed in 50 mM MES buffer (pH 6.7) and 8 mM MgCl$_2$. Pol I was assayed in 50 mM Tris (pH 7.5) and 10 mM MgCl$_2$.

The HeLa cell DNA polymerases were assayed according to Pedrali-Noy and Weissbach (1977) and Syvaoja et al. (1990). The mousel recombinant DNA polymerase was assayed according to Tanabe et al. (1979). The yeast DNA polymerases were assayed according to Burgers (1988).

DNAse I was assayed in 10 mM Tris, pH 8.8 at room temperature. Enzyme activity was determined by measuring the increase in absorption at 260 nm after adding 0.1 units of enzyme to 10 μg of DNA.

Alkaline phosphatase activity was measured according to Sigma Diagnostics procedure no. 104 (Sigma Chemical Co.).

Preparation of Arachidonic Acid Solutions:

Preparation of all arachidonic acid solutions was done at 0° C under a constant stream of Helium. Arachidonic acid (Sigma Chemical Co.) was prepared in 100% ethanol at a concentration of 100 mM and immediately diluted with 500 mM Tris (pH 9.6) to a concentration of 50 mM. Successive dilutions were made with 10 mM Tris (pH 7.5) so that the final concentration of ethanol in the assay was no more than 1 % (v/v).

Enzyme Inhibition by Fatty Acids:

Inhibition of the enzymes was assayed by pre-incubating approximately 0.02 units of enzyme and AA in ice for 30 minutes in a final volume of 15 μl (enzyme, AA solution, and water) and then adding the remaining reaction components and assaying as described above. The concentration range of AA used ranged from 1-500 μM, equivalent to a final concentration in the assay of 0.5-240 μM.

RESULTS AND DISCUSSION

AA inhibited the polymerization reaction only when added within the first five minutes. After the first five to ten minutes, the amount of inhibition decreased and the enzyme activity approached that of the control (Figure 1). These results suggest that AA acts by preventing the polymerase from binding to the template. DNA polymerase was not inhibited by AA, all other

DNA polymerizing enzymes were inhibited by AA, regardless of the source. The resistance of the M. luteus enzyme to inhibition may be due to unique structural or catalytic properties that allow the enzyme to catalyze the polymerizing reaction in the presence of AA.

Inhibition of the various classes of DNA polymerases from different sources by a range of AA concentrations.

Inhibition is expressed as percentage of a control *without* AA.

Source	Enzyme	% Inhibition of Enzyme Activity by:		
		100 μM AA	300 μM AA	500 μM AA
HeLa	pol α	100	100	100
HeLa	pol β	25.2	99.8	99.7
HeLa	pol γ	97.5	100	99.7
Mouse	pol β	5.3	22.7	94.2
Yeast	pol α	100	100	99.8
Yeast	pol δ	88	99.3	99.2
Yeast	pol ε	99.3	100	100
E. coli	pol I	100	100	100
M. luteus	pol	0	0	5.3

The inhibition of DNA polymerase activity by AA seems to be an enzyme-specific event since other enzymes such as DNAse I, trypsin, and alkaline phosphatase were not affected by this fatty acid (data not shown). This inhibition is more likely caused by a physical interaction of the enzyme and AA and not by oxidative damage, since it is fully reversible by dialysis (Figure 2). Furthermore, exposure of the enzyme to AA in the presence of antioxidants did not prevent inhibition, except at the highest concentrations tested, where a small decrease in the inhibitory activity of AA was observed, and is most likely due to dilution effects or changes in the pH.

This inhibition seems to be the result of a physical interaction between the enzyme and AA that prevents the polymerase from binding to the template DNA. AA will inhibit polymerization if exposed to the enzyme during a pre-incubation step or within the first five to ten minutes of the start of the reaction, but will not inhibit the reaction if added past the first 5-10 minutes. Preliminary data suggests that this physical interaction may be due to association of lipid micelles with the enzyme, based on the information obtained from Coomassie blue staining of non-denaturing PAGE and the apparent retardation of specific protein bands (data not shown).

AA was found to inhibit all the classes of DNA polymerases tested, with the notable exception of the Micrococcus luteus DNA polymerase. Since the E. coli pol I was found to be inhibited by the fatty acid, it cannot be concluded that the resistance of M. luteus pol to AA is merely due to a difference between prokaryotic and eukaryotic enzymes. It is possible that the M. luteus pol has important structural and possibly catalytic properties that differ from all the other enzymes tested and that the difference accounts for its resistance to AA. Unfortunately,

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this enzyme has not been thoroughly studied and detailed information about its structure and catalytic mechanism is lacking.

The insensitivity of other enzymes such as trypsin, alkaline phosphatase, and DNAse I to AA suggest a DNA polymerase-specific inhibition by this fatty acid. Although the concentrations of AA required for inhibition of the polymerases are relatively high and not likely to be encountered at normal physiological conditions, it is possible that a transient surge in the free AA concentration may occur within the cell during injury, early mitogenic stimulation or possibly cell division. For instance, it has been reported that D-glucose induces and accumulation of up to 35 mM nonesterified AA in pancreatic islet beta cells (Ramanandham et al., 1992).

In preparation for mitosis, the nuclear membrane must be dissolved in higher eukaryotes (Dingwall and Laskey, 1992), a process that may result in the hydrolysis of esterified fatty acids from the membrane. Inhibition of DNA pol activity by the released AA may be a mechanism by which the cell blocks any further use of energy in replication or DNA repair in order to conserve its life resources.

The inhibition of DNA polymerase activity by AA described herein may indicate a possible role for this fatty acid in the regulation of DNA replication. Furthermore, the somewhat high levels of AA required for inhibition may offer the possibility that an AA-related molecule may actually be a more potent inhibitor of DNA pol activity than the parent molecule. Novel AA-related compounds are now beginning to be identified as having potential physiological roles, which may result in the assignment of a whole new set of functions to AA and its metabolites. One of these could turn out to be the direct control of DNA replication through action on the polymerases.

Although enzymes from various sources were used in the study to obtain a multiple species analysis of the action of AA on the DNA polymerases, this was only an in vitro study. Its occurrence in vivo can only be speculated based on the results of this study, based on the assumption that the concentration of free AA in the cell can rise above basal levels during disease, cell division, injury, or toxic insult. No attempt was made to develop a detailed model of the underlying molecular mechanisms governing the interactions between AA and the enzymes.

Further research should focus on the quantitative determination of the minimum inhibitory AA concentrations in order to develop appropriate kinetic data to explain the mechanism of inhibition. Fluorescence measurements can be made in order to determine if micellar structures are associated with the DNA polymerases. Further experiments using radiolabelled AA may be of value in determining the extent, if any, of the association between AA and the DNA polymerases. Finally, other fatty acids or AA-related molecules could be tested to determine if these compounds are more potent inhibitors of the DNA polymerases.

CONCLUSION

- DNA polymerase activity could not be protected from inhibition by arachidonic acid with lipid binding protein, antioxidants, or by pre incubating the enzyme with DNA.

- AA solutions stored at 4° C for up to three weeks were equally inhibitory, independent of length of exposure to air.

    - Inhibition does not appear to be due to damage by oxidation.

- Inhibition of DNA polymerase was reversed by ultrafiltration dialysis.

    - Inhibition is reversible.

- Other enzymes were not inhibited by AA at the range of concentrations tested. DNAse I, alkaline phosphatase, and trypsin were completely resistant to inhibition by AA at concentrations up to 1 mM.

- Other DNA polymerases from various sources were also found to be inhibited by AA, with the exception of *M. luteus* DNA polymerase.

    - Inhibition by AA appears to be DNA polymerase-specific.

- Native gel electrophoresis of enzyme frations exposed to AA revealed an anomalous band pattern, indicating interference with migration.

    - DNA polymerase and AA seem to physically interact.

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Figure 9

Arachidonic Acid Cascade
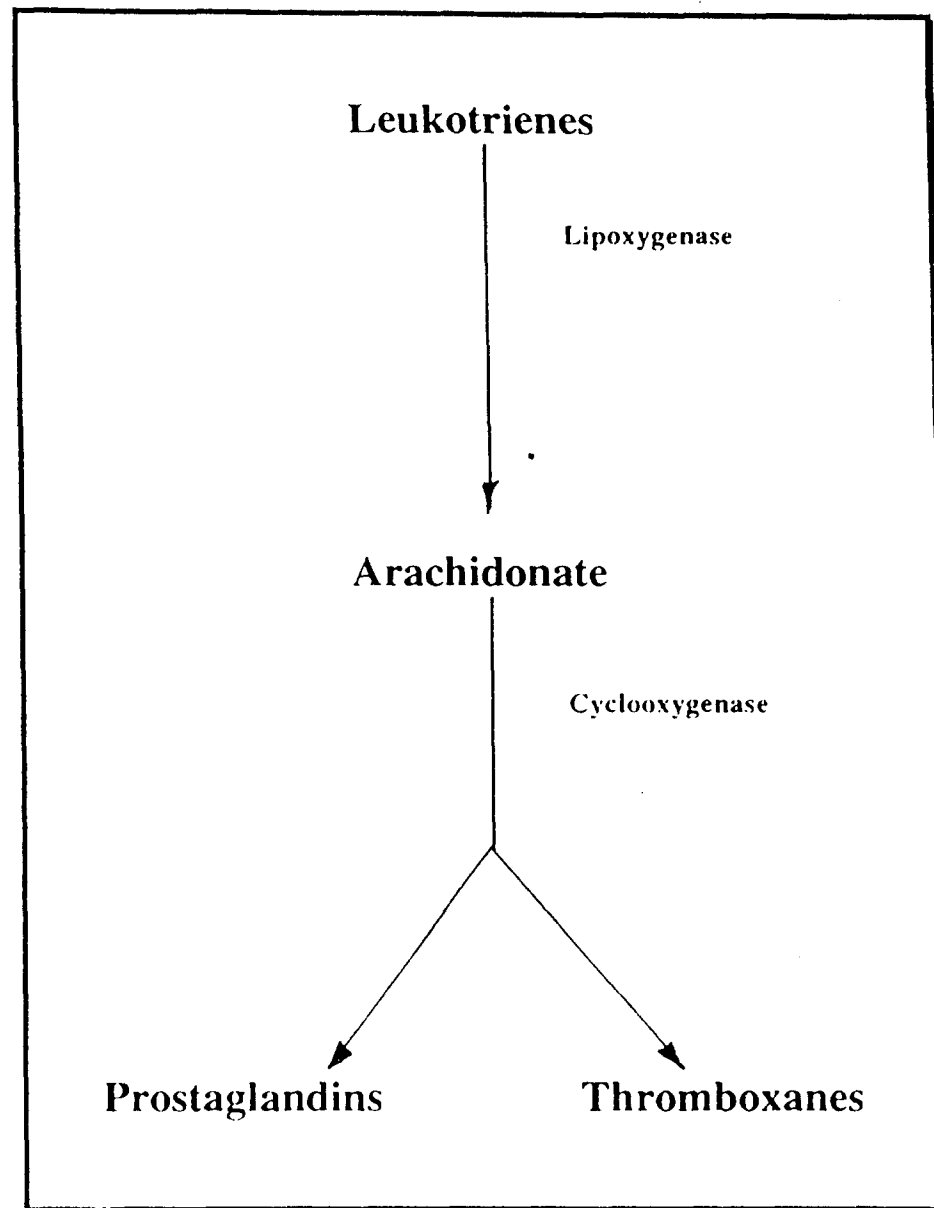
Arachidonate is the major precursor of eicosanoid hormones. Cyclooxygenase catalyzes the first step in a pathway leading to prostaglandins and thromboxanes. Lipoxygenase catalyzes the initial step in a pathway leading to leukotrienes. (Stryer, L. 1975. Biochemistry. W. H. Freeneu: NY, p. 991.)

Table 5

IC50 values for the inhibition of DNA polymerases by arachidonic acid

DNA polymerase Source	IC50μM
Leishmania mexicana Pol B	42
Chinese Hamster Ovary Pol A	84
Leishmania mexicana pol A	20
Calf Thymus pol α	106
Calf Thymus pol δ	Resistant
Microcucceis luteus	Resistant
M-MULV Reserve Transcriptase	<<50
AMV Reverse Transcriptase	175
E Coli DNA polymerase	460

Resistance of calf thymus DNA polymerase δ and Micrococcus luteus DNA polymerase to inhibition by arachidonic acid

arachidonic acid conc(μM)	% Enzyme Activity Remaining	
	calf thymus DNA polymerase δ	*Micrococcus luteus* poymerase
0	100	100
4	95	100
20	84	95
200	80	88
500	NT	77
2000	79	NT

**Figure 10.** Reversibility of the inhibition of CHO pol α activity by AA after dialysis by ultrafiltration.
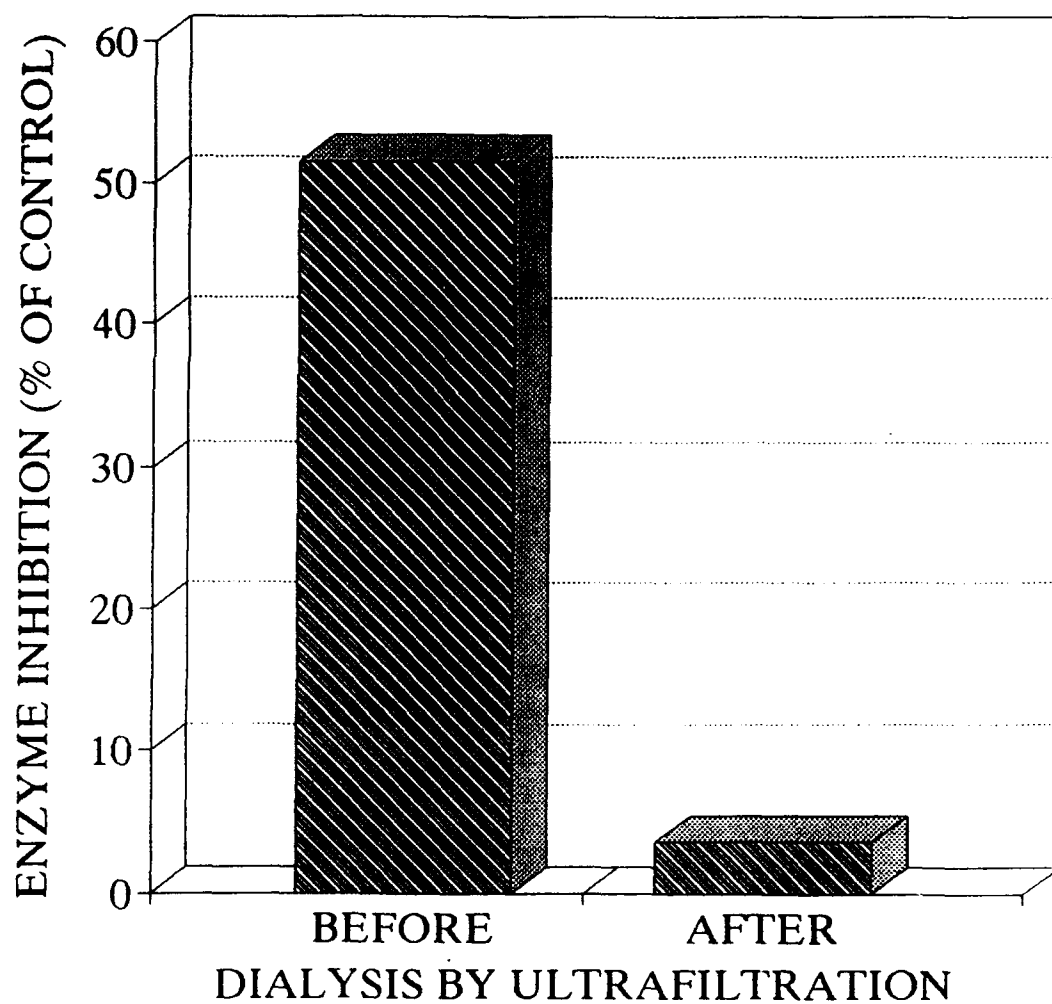
Figure 11

Protection with Antioxidants:

If inhibition is due to oxidation of the enzyme by lipid peroxides, the presence of antioxidants should offer protection against inhibition.

If lipid peroxides are involved, exposure of a freshly prepared solution of AA to air would result in significant inhibition after a prolonged exposure to air.

- Vitamins C and E, and butylated hydroxytoluene (BHT) did not fully protect the enzyme from inhibition, even at the highest concentrations tested.

- AA solutions were not altered in the amount of inhibition caused after a prolonged exposure to air at 4° C suggesting that oxidation does not play a role.
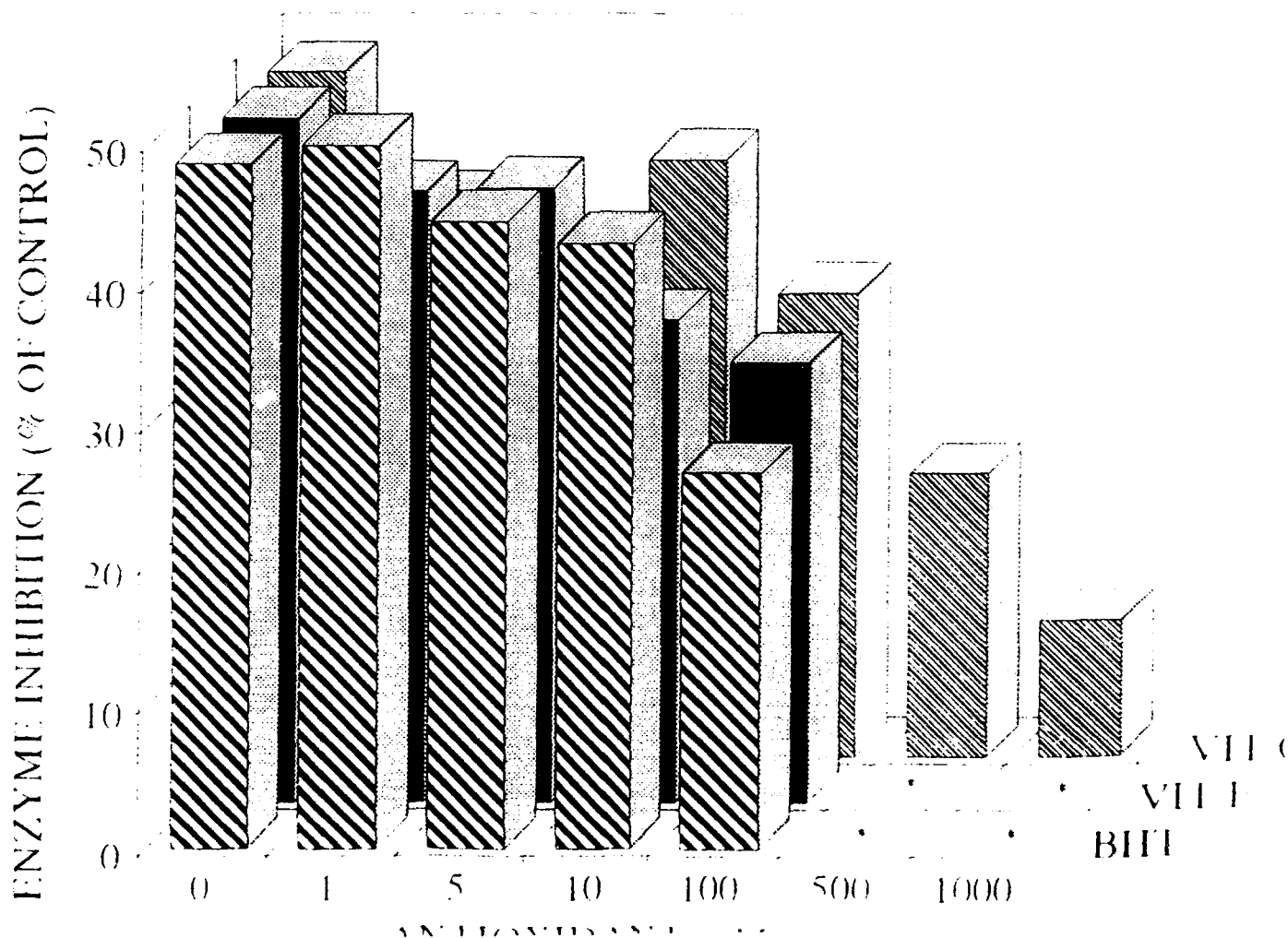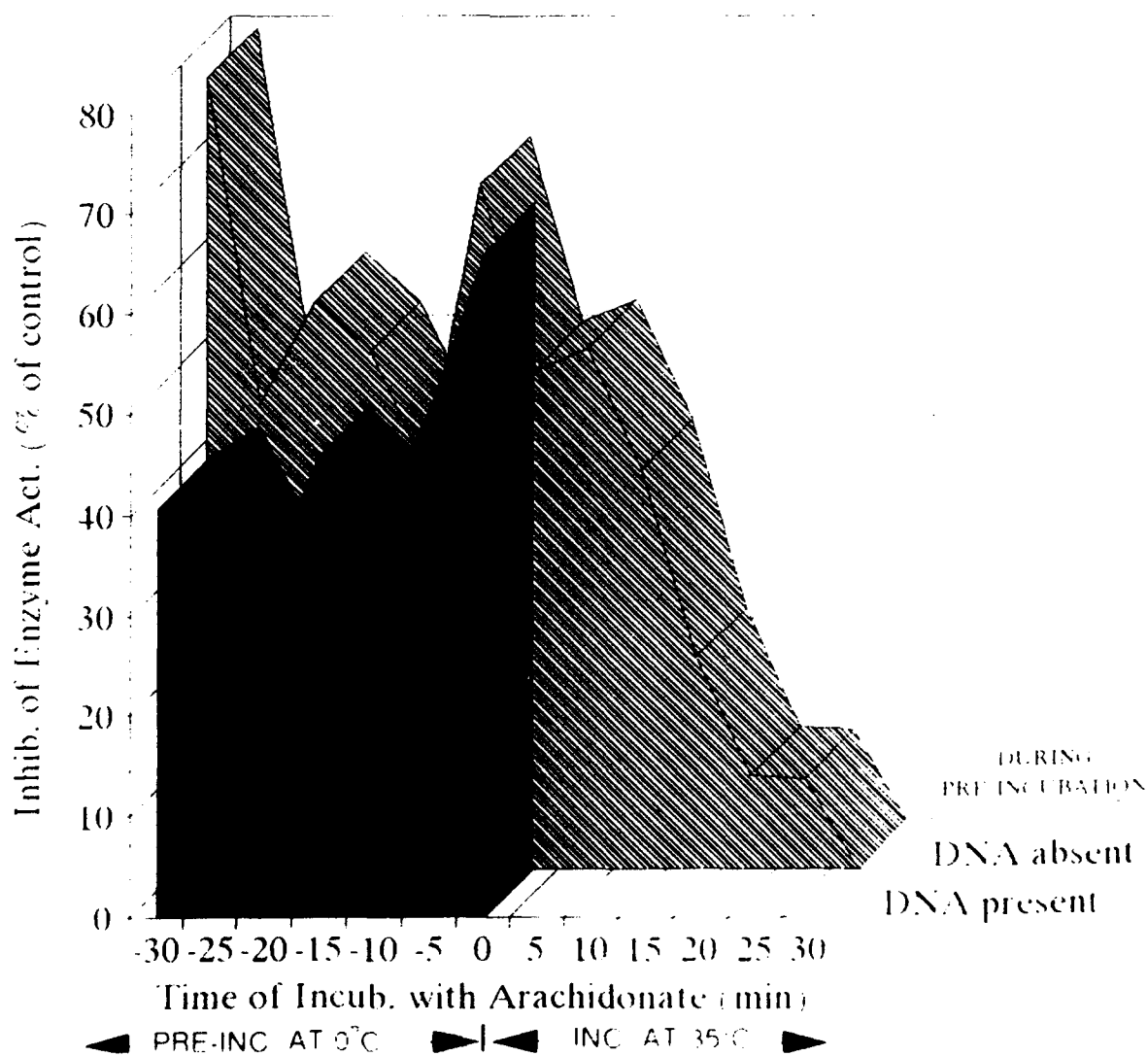
Figure 12

Exposure of Enzyme to DNA Template

If AA is sequestering or binding DNA polymerase and preventing it from attaching to the template, allowing the enzyme to bind to the DNA prior to adding AA would protect the enzyme from inhibition.

A time course experiment where AA was added at five minute intervals starting at 30 minutes prior to starting the reaction in the presence or absence of DNA, up to 30 minutes after starting the reaction showed that AA inhibits DNA pol if added during pre-incubation and up to 30 minutes after the start of the reaction. After 10 minutes of reaction, addition of AA did not inhibit the enzyme further. The presence of DNA template during the preincubation step did not protect the enzyme from inhibition.

Figure 13

Time course study. AA stops the polymerization reaction within the first five minutes. AA (100 μM was added at five minute intervals starting with the time when the CHO pol α was added (time = 0 minutes) up to 30 minutes after start as described in Materials and Methods.